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# Defectiveness of Interferon Production and of Rubella Virus Interference in a Line of African Green Monkey Kidney Cells (Vero)

JAN DESMYTER, JOSEPH L. MELNICK, AND WILLIAM E. RAWLS

*Department of Virology and Epidemiology, Baylor University College of Medicine, Houston, Texas 77025*

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Vero cells, a line of African green monkey kidney cells, failed to produce interferon when infected with Newcastle disease, Sendai, Sindbis, and rubella viruses, although the cells were sensitive to interferon. Further, infection of Vero cells with rubella virus did not result in interference with the replication of echovirus 11, Newcastle disease virus, or vesicular stomatitis virus, even in cultures where virtually every cell was infected with rubella virus. Under the same conditions, BSC-1 cells and other cells of primate origin produced interferon and showed rubella virus interference. The results indicate that the presence of rubella virus in the cell does not in itself exclude multiplication of other viruses and that rubella virus interference appears to be linked to the capability of the cell to produce interferon.

Cytopathic changes are slight or absent in many kinds of mammalian cells infected with rubella virus, but the virus conveys resistance to superinfection with a large number of unrelated viruses (16). The interference does not affect the uptake and eclipse of the challenge viruses and thus resides at an intracellular level (11, 21). The interferon system represents the only known mechanism of heterologous viral interference which is characterized by these properties, and interferon has been found in tissue cultures infected with rubella virus (15, 17). These and other analogies have led some authors to suggest that rubella virus interference is mediated by the interferon system (5, 15, 26). However, other reports have indicated that the rubella virus genome may be directly responsible for the interference with Newcastle disease virus (10, 11). There also is the possibility that the activity of an interferon system in cells infected with rubella virus is merely coincidental or ancillary. The mechanism of rubella virus interference, whether or not it is mediated by the host cell genome, could be unrelated to interferon. The use of cells which are defective in the production of interferon or are insensitive to interferon could provide a clue as to whether rubella virus interference is basically dependent upon the interferon system. This study reports the finding that a stable line of African green monkey kidney cells (Vero) is defective in its production of interferon but is sensitive to the action of interferon, and describes the replication of certain viruses in such cells coinfected with rubella virus.

## MATERIALS AND METHODS

*Tissue culture.* Vero cells, a stable line of African green monkey kidney cells (27), were kindly supplied by J. S. Rhim of Microbiological Associates, Bethesda, Md. (22) in their 140th passage and were used after 5 to 15 additional passages. BSC-1 cells (8), another stable line of African green monkey kidney cells, were obtained from laboratory stocks (19). Newborn foreskin fibroblasts were grown as described (4) and were used between passages 10 and 20. Primary chick embryo cell cultures were prepared from 10-day-old embryonated eggs. Primary African green monkey kidney cells (pGMK) were grown as described (13). With the exception of the latter cells (13), the growth medium consisted of Eagle's medium supplemented with 0.75 g of sodium bicarbonate per liter, 10% fetal bovine serum, 100 units of penicillin per ml, 100 $\mu$g of kanamycin per ml, and 50 units of mycostatin per ml. In maintenance medium, 1.5 g of sodium bicarbonate per ml and 2% fetal bovine serum were substituted. Agar overlays, 4 ml per 6-cm plastic petri dish, contained the same constituents as the growth medium plus 1.2% Difco agar and 25 mg of neutral red per liter. The sodium bicarbonate content of the medium of all cultures incubated in a 5% $CO_2$ atmosphere was 2.25 g/liter. The incubation temperature was 37 C.

*Viruses.* Rubella virus R-1, isolated from thyroid tissue culture of an infant with congenital rubella (21) and prepared in $BHK_{21}$ cells (24), was assayed in BSC-1 cells by a previously published modification (20) of the hemadsorption-negative plaque test (10). Echovirus 11, Gregory strain, was grown in pGMK cells and assayed by production of cytopathic effect (CPE) in the same cells. Vesicular stomatitis virus (VSV), Indiana serotype, was grown in pGMK cells and assayed in BSC-1 cells by plaque formation, and

also in the other kinds of cells when used for interferon titration. Newcastle disease virus (NDV), California strain, was obtained as the allantoic fluid harvested 2 days after infection of 11-day-old chick embryos and was assayed by plaque formation on 2-day-old monolayers of chick embryo cells. Sendai virus, strain 52, was grown in a similar manner and assayed by production of hemadsorption in pGMK cells. Sindbis virus, strain Ar-339, was grown in chick embryo cells and assayed in a manner similar to NDV.

*Production and assay of interferon.* Except when mentioned otherwise, interferon was prepared by inoculating cell monolayers in 16-oz (80 $cm^2$) bottles with 1 plaque-forming unit (PFU) of NDV per cell contained in 30 ml of maintenance medium without serum. The fluids were harvested after 36 to 48 hr of incubation in a 5% $CO_2$ atmosphere. This procedure is similar to that described by Merigan et al. (14) for human foreskin interferon. Pooled fluids were acidified to *p*H 2.0 with HCl, stored 5 days at 4 C, brought to *p*H 7.0 with NaOH, subjected to low-speed centrifugation, and then centrifuged twice for 2 hr at 100,000 × *g*. The supernatant preparations were stored frozen at −20 C. Interferon assays were carried out on BSC-1 cell monolayers in petri dishes or on other cells where indicated. A 4-ml amount of test fluid diluted in maintenance medium was left in contact with the cells for 18 hr in a 5% $CO_2$ atmosphere. The cells were then washed once with medium without serum, or three times, in the case of interferon induced by Sindbis virus. Fifty to 80 PFU of VSV in 0.2 ml was allowed to adsorb for 1 hr, then agar overlay was applied. This procedure is similar to that described by Petralli et al. (18) for the assay of human interferon in foreskin cells. Plaques were counted after 2 days. The interferon titer is expressed as the highest twofold dilution giving at least 50% plaque reduction. The reciprocal of this value is the number of interferon units per ml. Two or three replicate plates were used for each dilution tested. Interferon activity was considered absent when, at a dilution of 1:8, there was less than 50% reduction in the number of VSV plaques.

## RESULTS

*Defectiveness of interferon production in Vero cells.* The Vero cell line was selected for study as a possible candidate for a defective interferon system. The defective system was suspected because a small inoculum of rubella virus in pGMK fails to replicate to maximal titers [which has been explained (26) by the activity of endogenous interferon], whereas the virus grows to high titers in Vero cells regardless of the size of the inoculum (9). BSC-1 cells (rather than pGMK) were selected for control purposes, because it was found that the response of BSC-1 cells to interferon and their production of interferon was comparable to that of pGMK, and this cell line gave more consistent results than did different batches of pGMK cells. In addition, BSC-1 cells support the replication of rubella virus as demonstrated by interference to superinfection with other viruses (16, 20).

TABLE 1. *Induction of interferon in Vero, BSC-1, and human foreskin (FSK) cells with Newcastle disease virus*

| Interferon source | Assay cells | | |
|---|---|---|---|
| | Vero | BSC-1 | FSK |
| Vero Expt 1 | 0[a] | 0 | 0 |
| | 0 | 0 | ND |
| 2 | 0 | 0 | 0 |
| | ND[b] | 0 | ND |
| BSC Expt 1 | 128[c] | 512 | 512 |
| | 128 | 256 | 512 |
| 2 | 64 | 256 | 1,024 |
| | 128 | 512 | 256 |
| 3 | 128 | 256 | 512 |
| FSK Expt 1 | 64 | 128 | 128 |
| 2 | 64 | 64 | 256 |

[a] <1:8.
[b] Not done.
[c] Units of interferon per milliliter.

The results of comparative attempts to produce interferon with NDV in Vero, BSC-1, and foreskin cells and to assay interferon in the same cells are given in Table 1. No interferon was detectable in fluid from Vero cultures at a 1:8 or higher dilution, whether tested on Vero, BSC-1, or human foreskin cells. BSC-1 cells were slightly better producers of interferon than foreskin cells, whereas foreskin cells were slightly more sensitive to interferon. Vero cells, however, were sensitive to interferon produced in BSC-1 or foreskin cells, but slightly less than BSC-1 cells. As expected (1, 6), no indication of species specificity between human and monkey interferon was found. Similar results were obtained in other experiments with BSC-1 and Vero cells. Five similar attempts to produce interferon in Vero cells failed to reveal activity at the lowest dilution tested (1:8 or 1:16). Differences in the capacity of VSV to replicate in the assay cells could not explain the results obtained. The average plaquing efficiency of the VSV stock was 0.8 in Vero cells and 0.3 in foreskin cells when compared to BSC-1 cells. Plaques could be conveniently counted in both Vero and BSC-1 cells after 36 hr, and 12 to 18 hr later in foreskin cells. No gross differences were observed between Vero and BSC-1 cells in the time at which plaques appeared and increased in number and size.

Development of NDV cytopathic effect was

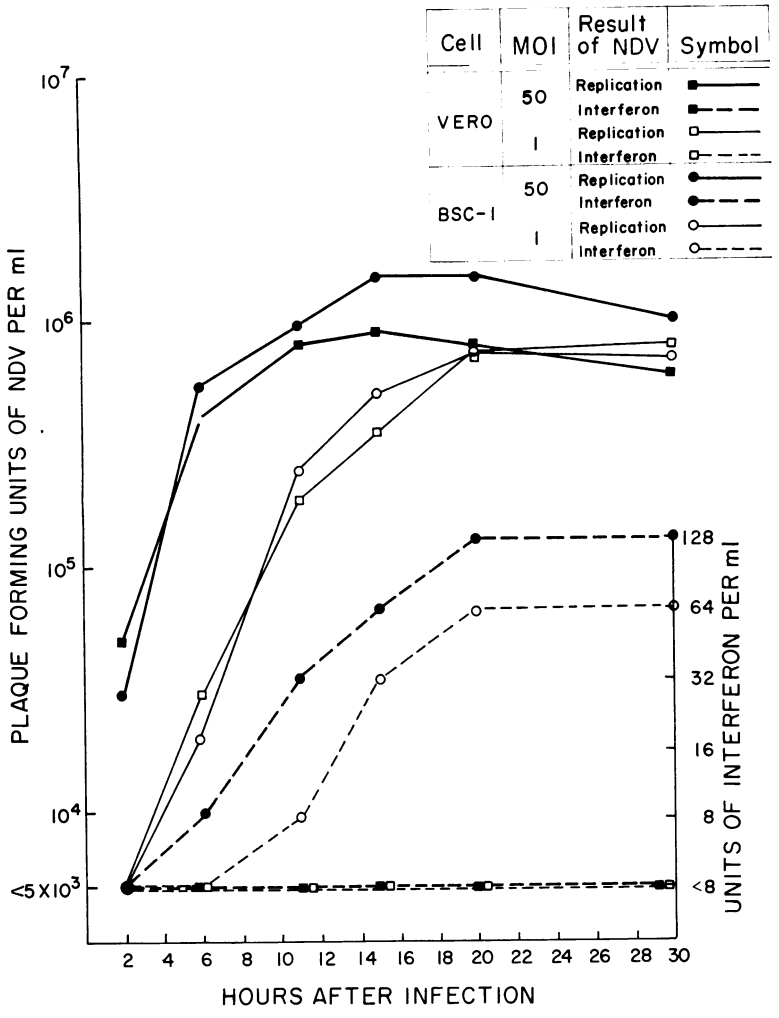


FIG. 1. *Single-cycle multiplication of Newcastle disease virus and production of interferon in Vero and BSC-1 cells, 200,000 per tube monolayer in 1 ml.*

somewhat more rapid in Vero cells than in the other cells. Interferon activity was therefore examined in fluids harvested 24 hr after infection of Vero cells rather than at 48 hr. Again, no activity was found. After challenge of Vero cells with NDV at a multiplicity of infection (MOI) of 20 and 0.05 rather than 1, or with ultraviolet-inactivated NDV (4), no interferon activity was found. In Fig. 1, the single-cycle multiplication of NDV and the appearance of interferon in tube cultures of Vero and BSC-1 cells are represented. Monolayers of $2 \times 10^5$ cells were infected at an MOI of 1 and 50 PFU of NDV in 0.2 ml; after 2 hr, they were washed five times, 1 ml of medium was added, and the cultures were harvested at different times of incubation. For each harvest, four replicate tubes were frozen and thawed three times, pooled, assayed for NDV, and also processed for assay of interferon. The replication of NDV in Vero and BSC-1 cells was remarkably similar; however, at no time was interferon found in Vero cultures. The inhibitor produced in BSC-1 cells was established as interferon by most of the criteria applied to human foreskin interferon (3), including insensitivity to NDV antiserum and absence of activity on chick cells. Foreskin and BSC-1 interferons were active on rabbit kidney cells (3), whereas comparable Vero fluids were not.

Although NDV is regarded as the most useful agent for producing interferon in high yield (25), other viruses reported to be satisfactory inducers

were tested. Sendai and Sindbis viruses were used under the same conditions as NDV. They produced more rapid CPE and fluids were therefore harvested after 24 hr. Acid inactivation of these viruses was limited to 2 days. No interferon activity was found in Vero fluids, whereas interferon titers of 1:512 and 1:64 were obtained in fluids of BSC-1 cells infected with Sendai virus, and of 1:32 and 1:16 in BSC-1 cells infected with Sindbis virus. Interferon was absent in fluids of Vero cells infected 2 or 4 days earlier with rubella virus at different MOI, but interferon was also not well demonstrable in similarly treated BSC-1 cells (titer 1:8 or, more frequently, < 1:8 on BSC-1 or foreskin cells).

The possibility was considered that Vero cells may produce interferon and simultaneously produce an interferon antagonist which is active in primate cells. Such a situation has been shown to exist between different types of murine cells (23). Different dilutions of interferon induced by NDV in BSC-1 cells were therefore made in undiluted Vero NDV-"interferon," and assayed on BSC-1 cells. It was found that the titer of the interferon induced in BSC-1 cells was normally expressed, indicating that an interferon antagonist was not produced by the Vero cell. It is concluded from the above experiments that Vero cells are defective in their ability to produce interferon; however, these cells are sensitive to exogenously added interferon.

*Defectiveness of rubella virus interference in Vero cells.* Rubella virus was titrated by the hemadsorption-negative plaque method in BSC-1 cells (20), by development of CPE in Vero cell tube cultures (9, 22), and by interference with the development of echovirus CPE in BSC-1 cells as originally described in pGMK cells (16). The titers obtained in repeat titrations by the three methods were similar ($10^{4.8}$ to $10^{5.2}$ infectious units per ml). This was predicted, since it has been shown that the hemadsorption-negative plaque titer (10), as well as the titer obtained by development of CPE in Vero cells (9), are similar to the titer of interference with echovirus CPE in pGMK cells. When Vero cells infected with rubella virus were challenged with 1,000 $TCD_{50}$ of echovirus, there was complete destruction of the cell sheet which occurred rapidly and concurrently in all tubes, whether they had been inoculated 5 to 7 days earlier with low or with high dilutions of the rubella virus preparations. At the moment of challenge with echovirus, Vero cell monolayers containing rubella virus showed distinct but limited CPE when compared to uninoculated controls. The rubella virus CPE did not affect the continuity of the cell sheet nor did it interfere with the development of typical enterovirus CPE.

*NDV.* Attempts were made to demonstrate infection of Vero cells with rubella virus by the formation of hemadsorption-negative plaques upon challenge with NDV (20). Vero cell monolayers in petri dishes were inoculated with different dilutions of rubella virus, incubated under fluid medium, and challenged with NDV after 1, 2, 3, and 4 days. Since NDV hemadsorption was found to develop at about the same rate in noninfected Vero cells as in BSC-1 (20) and in pGMK (10) cells, erythrocytes were added at the same time as to the other cell types, i.e., after 15 to 18 hr. No hemadsorption-negative plaques or inhibition of NDV hemadsorption was seen in Vero cells infected with rubella virus. Sheep erythrocytes were adsorbed to Vero cell monolayers whether or not they were infected with rubella virus and without regard to the size of the rubella virus inoculum or of the time they had been infected. Treatment with NDV antiserum after adsorption of NDV, designed to avoid spurious binding of erythrocytes (10), did not result in the appearance of plaques. Hemadsorption-negative plaques developed in control cells, which included pGMK, BSC-1, human embryonic fibroblasts, and three strains of human foreskin cells, and in CV-1 cells, another stable line of African green monkey kidney origin.

The above results with NDV and echovirus suggested that challenge virus may multiply in single Vero cells which were coinfected with rubella virus. However, an alternative explanation would be that relatively few Vero cells were infected with rubella virus at any time and with any inoculum. The following experiments were performed in Vero cells to establish and to measure maximal infection with rubella virus.

Vero cell monolayers were infected with rubella virus at an MOI of 0.1 to 1. After 4 days of incubation, part of the cultures was infected with NDV at an MOI of 10 and further processed for the single-cell hemadsorption test, as described by Marcus and Carver (11). The percentage of cells hemadsorbing erythrocytes was estimated by counting 200 cells. The other part of the cultures was used to establish the percentage of cells infected with rubella virus by infectious center assay (21; Rawls, Desmyter and Melnick, *in press*). The results are given in Table 2. Essentially all Vero cells under the given conditions were apparently infected with rubella virus, and virtually all such cells allowed development of NDV hemadsorption, thus proving coinfection of individual Vero cells with rubella virus and NDV. The development of NDV hemadsorption was measured by the single-cell technique in Vero and BSC-1 cells; some were coinfected with rubella virus under conditions of maximal infection and some were not (Table 3). Vero cells

TABLE 2. *Coinfection of Vero cells with rubella virus and Newcastle disease virus*[a]

| Expt no. | Cells with NDV hemadsorption (%) | Determination of rubella infection of Vero cells | | |
|---|---|---|---|---|
| | | Estimated cells per tube | Tubes with rubella/ tubes tested | $InD_{50}$ (cells)[b] |
| 1 | 97 | 3[c] | 4/5 | |
| | | 1 | 4/5 | 0.8 |
| | | 0.3 | 0/4 | |
| 2 | 98 | 3 | 5/5 | |
| | | 1 | 2/5 | 1.3 |
| | | 0.3 | 0/3 | |
| 3 | 98 | 3 | 4/5 | |
| | | 1 | 3/5 | 0.9 |
| | | 0.3 | 1/5 | |

[a] Vero cells were infected with rubella virus and incubated 4 days at 37 C. The cultures were superinfected with NDV and the per cent of hemadsorbing cells was determined after 15 to 18 hr of additional incubation. Replicate cultures were used to determine the per cent of cells infected with rubella virus by infectious center assay.

[b] Inhibitory $dose_{50}$ calculated by method of Reed and Muench.

[c] When tubes contained 10 and 100 cells, four of four tubes tested were found to contain rubella virus in all experiments.

produced NDV hemadsorption whether or not they were infected with rubella virus; in BSC-1 cells, NDV hemadsorption was markedly inhibited in the coinfected cells. Vero and BSC-1 cells which were not infected with NDV did not adsorb sheep erythrocytes.

*VSV.* The interference of rubella virus with VSV was determined by examining virus replication and plaque formation in Vero and BSC-1 cells coinfected with rubella virus. Vero and BSC-1 cells maximally infected with rubella virus and control cells were infected at a high and low MOI of VSV and washed after 2 hr of adsorption. The yields of VSV after 36 hr of incubation of the cultures are given in Table 4. By infectious center assay, it was determined that over 90% of the BSC-1 and Vero cells were infected with rubella virus, yet the replication of VSV in Vero cells infected with rubella virus was more than a 1,000-fold greater than in BSC-1 cells infected with rubella virus. When a VSV preparation was titered in monolayers of the same sets of cells, a titer of $1.3 \times 10^7$ PFU/ml was obtained in noninfected Vero cells and $1.0 \times 10^7$ PFU/ml in Vero cells infected with rubella virus. The plaque characteristics were comparable in both infected and noninfected monolayers. In contrast, a titer of $2.7 \times 10^7$ PFU/ml was obtained in noninfected BSC-1 cells, whereas no plaques (less than 500 PFU/ml) appeared in the rubella-infected BSC-1 cells.

The experiments with echovirus, NDV, and VSV demonstrated that rubella virus interference is defective in Vero cells. This defectiveness appeared to operate at the level of the single infected cell. Neither the presence of rubella virus synthesis nor the ultimate, if delayed, cytolytic effect of rubella virus in the Vero cell seemed to interfere with the replication of challenge virus.

## DISCUSSION

Although cells which are defective in interferon production or are insensitive to interferon may have considerable potential in the elucidation of viral functions related to interferon, no intensive search for such cells has been made in recent years. Results of earlier studies concerning the relative efficiency of production and response to interferon by various types of cells have been summarized by Ho (7). The described defectiveness in interferon production upon viral challenge of Vero cells is the first instance in which monkey

TABLE 3. *Development of NDV hemadsorption in Vero and BSC-1 cells infected or not infected with rubella virus*[a]

| Expt no. | Vero cells | | BSC-1 cells | |
|---|---|---|---|---|
| | With rubella | Without rubella | With rubella | Without rubella |
| 1 | 97[b] | 99 | 1.5 | 100 |
| 2 | 98 | 100 | 0 | 98 |

[a] Vero and BSC-1 cells were infected with rubella virus and incubated 4 days; then they were superinfected with NDV and examined for the number of cells which developed hemadsorption.

[b] Percentage of cells in which NDV induced hemadsorption of sheep erythrocytes.

TABLE 4. *Rubella virus interference with multiplication of vesicular stomatitis virus in Vero and BSC-1 cells*

| Cell types | Infected with rubella virus[a] | VSV (MOI 0.001) | VSV (MOI 20) |
|---|---|---|---|
| Vero cells..... | − | $1.3 \times 10^7$[b] | $1.5 \times 10^7$ |
| Vero cells..... | + | $1.0 \times 10^7$ | $0.3 \times 10^7$ |
| BSC-1 cells.... | − | $2.7 \times 10^7$ | $1.0 \times 10^7$ |
| BSC-1 cells.... | + | $<5 \times 10^2$ | $1.0 \times 10^3$ |

[a] Cells were infected with rubella virus and incubated 4 days; then they were superinfected with VSV and harvested after 36 hr.

[b] Number of PFU obtained per 200,000 cells grown in 1 ml of medium.

cells seem to be qualitatively defective in this function. Obviously, the data presented do not rule out the genetic capability of Vero cells to code for interferon (2), nor has the production of interferon in amounts too small for detection been excluded. However, the data strongly suggest loss of, or interference with, the transcription or translation of the genetic information which codes for interferon production. The sensitivity of Vero cells to exogenous interferon supports the concept that the afferent and efferent pathways of the interferon system are controlled by different genetic loci.

Rubella virus is unusual among human viruses in its lack of production of CPE coupled with interference with the replication of a large number of unrelated viruses. It would seem that a single intracellular mechanism is responsible for such interference in different cells and with different viruses. Rubella virus interference may be due to any of the following mechanisms. (i) It may be conveyed by the virus genome, the host cell genome playing no part. Studies of rubella virus interference with NDV replication in pGMK cells using dactinomycin (10, 11), an inhibitor which essentially blocks DNA transcription, supports the conclusion that such a mechanism is indeed operative in this case. In Vero cells, however, NDV hemadsorption develops equally well whether or not the cells are coinfected with rubella virus. The data indicate that the presence of replicating rubella virus does not in itself exclude the replication of NDV and that rubella virus interference is not solely a function of the rubella virus genome. The loss of rubella virus interference to NDV replication in pGMK cells, when the rubella-infected cells were dispersed by trypsinization (11), is compatible with this conclusion. (ii) The interference could be mediated by the host cell genome in a manner unrelated to interferon. (iii) The interference could be mediated by the host cell genome through the interferon system; that is, rubella virus may convey interference by the production of interferon or by inducing any other step in the interferon pathway leading to the production of translation-inhibitory protein (12). Studies of rubella virus interference with VSV and poliovirus in pGMK cells combined with the use of dactinomycin (26) have led to the conclusion that rubella virus interference in most, if not in all, instances is mediated by interferon. However, the data upon which this conclusion was drawn are compatible with any mechanism involving transcription of the host cell genome, and the interferon system could either provide the sole mechanism of interference or act as an auxiliary mechanism. The simultaneous occurrence of the two unusual characteristics of defectiveness in interferon production and absence of rubella virus interference in Vero cells is highly suggestive of a causal relationship between the two properties. A definite clarification of the problem awaits tools to study production, fate, and activity of interferon at the level of the same cell. The present data, however, bring support to the hypothesis that rubella virus interference is mediated through the interferon pathway and at the initial step of interferon production.

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